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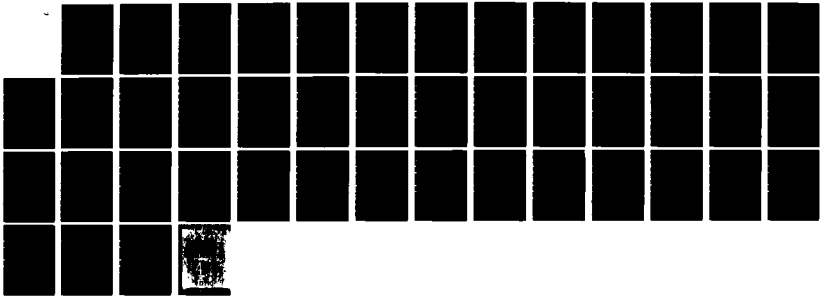

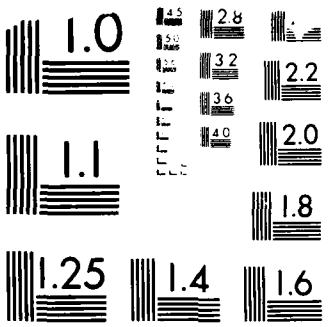

FATTY ACID METABOLISM AND PULMONARY INSUFFICIENCY STUDIES

Joseph A. Moylan, M.D.

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

During the first year of the project a reproducible free fatty acid analysis was carried out with a total of 1407 studies being performed. The results of correlating this to respiratory distress in trauma patients revealed a trend correlating elevated free fatty acid levels with PO_2-FiO_2 ratios of 1.9, indicating respiratory distress.
Patients with normal PO_2-FiO_2 ratios had relatively normal free fatty acid levels following injury.

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Animal studies were carried out during the first year showing a reduction of both cytochrome P-450 and mixed function amino oxidase (MFAO) activities following injury. Injury produces an elevation in free fatty acid levels in these animals.

SUMMARY

It can be seen in the attached data that many of the long-term aims of this project, which were anticipated to cover the first two years, have been accomplished in the first year. This includes the development of an accurate and rapid method for quantitative and qualitative fatty acid analysis. The initial correlation of the relationship of unbound fatty acids to post-traumatic respiratory distress shows that there is a definite trend in this relationship and that statistical correlation can be accomplished in the near future by increasing the number of patient studies. Animal studies have been carried out to define the basic metabolic effect of trauma on lung and liver dysfunction, evaluating cytochrome P-450 and mixed function amino oxidase activities, particularly. These studies were undertaken to provide base line measurements of the effect of trauma and NEFA, specifically on these organs. Measurement of free fatty acid levels in these animals following injury are consistent with the elevation seen in humans. Further work following this base line investigation will include specific fatty acid administration to compare the mechanism of cell dysfunction which we think will be similar.

The end benefits of these investigations are pertinent to military medical efforts both in the treatment of multisystem injury following combat and during peace time vehicular accidents as well as the influence of injury and disease processes on drug and specific organ metabolism. We have seen differences in these investigations in young and old animal experiments which may be pertinent to the young military recruit.

TABLE OF CONTENTS

A. INTRODUCTION

Respiratory distress is a major problem in multisystem injured patients as well as other severely stressed individuals. The relationship between fat emboli, increased serum fatty acid levels and pulmonary dysfunction has been poorly defined because of many limitations. Quantitative and qualitative analysis of serum fatty acids have been irreproducible from laboratory to laboratory limiting the ability to define the relationship of these serum substances with disease process. In addition, the actual pathophysiology of fatty acids on cellular and molecular dysfunction has never been investigated indepth. The short term goal of this project during the first year was to (1) develope a reproducible fatty acid analysis, (2) develope an animal trauma model leading to increased free fatty acid levels and (3) to evaluate the potential toxic effects of free fatty acids *in vivo* and *in vitro* on enzyme and cellular function.

I. BACKGROUND

Laboratory investigations have shown that injections of free fatty acids such as oleic or arachadonic produced pulmonary decompensation in a variety of animals. Incubation and binding of free fatty acids to albumin prior to injection has eliminated the toxic effects of these agents on the pulmonary function. Other experimental work has demonstrated toxic effects of fatty acids on other cells in the body such as platelets and myocardial function.

In the normal state, free fatty acids are transported through the intravascular space in a bound state with albumin being the primary binder. Following injury, however, there is significant elevation in free fatty acid levels in patient incurring these injuries with the degree of elevation proportional to the magnitude of injury.

Carlson and his co-workers linked this excessive lipid mobilization with microscopic pathological picture in animals consistent with clinical fat emboli in man. Studies from our laboratories have shown the albumin levels in patients developing fat emboli syndrome to be significant lower in comparison to the patients who remained asymptomatic, allowing a significant portion of fatty acids to circulate in the unbound or toxic state. Prior quantitative analyses have attempted to compare total free fatty acids levels in patients without defining either specific types or the degree of binding. Most long chain fatty acids are bound to the primary binding sites on serum albumin, while drugs and other short chain fatty acids are attached to the secondary and tertiary binding sites. Since prior extraction procedures have produced significant shifts in the equilibrium in the direction of the unbound fraction, the true percentage of circulating unbound free fatty acid could not be determined. Our goal was to overcome these problems in the analytical component of our laboratory investigation.

In investigating the pathophysiology of these agents in organ dysfunction following injury, prior animal studies have evaluated the association of free fatty acids and trauma with whole organ system failure. Di Augustine and Fouts have shown that unsaturated fatty acids inhibited hepatic microsomal metabolism of type I drugs with inhibition increasing with unsaturation: arachidonic > linolenic > linoleic > oleic acids. In addition, they concluded that the peroxidase process may play a role in drug induced changes consistent with fatty liver. However, the cumulative effect and the relationship of trauma and free fatty acids on cell dysfunction and drug metabolism has not been completely investigated. Using Bath electron paramagnetic techniques for in vivo whole cell function and in vitro micro-

zome enzyme systems could be effective in relating the metabolic changes secondary to specific biochemical alterations from fatty acid toxicity. We have chosen cytochrome P-450 system which is a visible wavelength spechtrophotometric quantilization of enzyme reaction products and mixed function amine oxidase to evaluate these changes.

B. LONG TERM SPECIFIC AIMS OVER 4 to 5 YEARS

I. Patient Studies:

a. To develop an accurate and rapid method to quantitate and qualitate fatty acids in plasma samples.

b. To define the relationship of unbound fatty acids and post traumatic respiratory distress.

c. To identify specific fatty acids with increased toxicity in trauma patients.

II. Animal Studies:

a. To evaluate the cellular metabolic effects of trauma and increase fatty acids on lung and liver function using drug metabolism as a parameter of cell function.

b. To define the molecular mechanism of cell dysfunction following fatty acid administration.

c. To correlate cellular function changes in whole cell preparation and microsomal enzyme preparation produced by fatty acid administration.

d. To evaluate the specific fatty acids with increased toxicity in humans on whole cell culture preparations and microsomal enzyme preparations.

C. ACHIEVEMENT OF AIMS

I. Development of Method for Quantitation of Plasma Free Fatty Acids

A. Introduction

The complex and confusing relation between nonesterified free fatty acids (NEFA) and different types of physiological stress has been hampered by the lack of reliable and sensitive means of measuring total and individual NEFA levels in plasma. We believe that a number of literature reports on NEFA measurements are unreliable due to the methods used. In our development of a satisfactory analytical technique, we have been able to identify the types of errors involved in colorimetric procedures and in certain aspects of collection, storage, extraction, and measurement of NEFA. For these reasons, we believe many of the NEFA values reported in the literature must be viewed quite critically.

B. Collection and Extraction of Blood Samples

Experience with several different blood samples on different occasions has shown that citrate, heparin, and EDTA and equally effective anticoagulants. Aliquot plasma samples using these agents have virtually identifical NEFA totals as measured by gas chromatography. It was also found that if the blood samples were not immediately chilled after collection, but remained at room temperature for up to 1 hr. in either heparin or citrate, NEFA levels were not affected. This is in contrast to several reports, including Sampson and Hensley (1975), but in agreement with Rogiers (1978).

The extraction method is critical. It has been found, for example, that efficient extraction of NEFA is achieved with hexane: chloroform:methanol. Methanol is necessary in amounts 2-5% in order to raise extraction efficiency from approximately 60% to an average of approximately 90%. We found that 5% methanol resulted in the extraction

of larger amounts of trigylcerides, cholesterol, and other lipid class compounds without noticeably increasing NEFA extraction efficiency.

Use of an internal standard, in our case heptadecanoic acid, appears to be the best means of dealing with daily variations in volumetric techniques, extraction efficiency, and injection techniques. This acid is not found in human plasma and is eluted close to the peaks of interest. However, the internal standard must be added to the plasma and well mixed, rather than added to the extraction solvent. Equilibration with the plasma is complete within one minute, as we showed in a kinetic study by mixing C^{14}-palmitate with plasma and measuring extraction efficiency.

C. Measurement of NEFA

1. Colorimetric methods for measuring NEFA have been reported by several workers. Using a sensitive method developed by Itaya (1976), we have compared a wide range of plasma samples by taking aliquots and measuring NEFA by the colorimetric and gas chromatographic methods. In all instances, the colorimetric method yielded results 10 to several hundred percent higher than the GC method. Apparently, class of compounds other than NEFA are extracted with the hexane:chloroform:methanol mixture, leading to interference. This is especially true, we find, for patients who have received heparin.

2. Although direct measurement of NEFA after extraction from plasma would shorten analysis time, we find that present day GC column liquid phases do not meet our requirement for separation and sensitivity. For example, the 10% SP216 P5 cyanosilicone column used by Sampson and Hensley (1975) is practical for analysis of the C-18 fatty acids, but there is significant tailing. Other fatty acids of interest, such as arachadonic acid, are not satisfactorily resolved.

For this reason, we at present find that GC analysis of NEFA as the methyl esters is the most satisfactory method to date.

3. We have chosen diazomethane as the ideal methylation method for NEFA esterification. It is a procedure requiring mild conditions and is essentially 100% complete within minutes. We compared several plasma samples by methylating twice with diazomethane and analyzing by GC after each methylation. Results were the same. an additional check, we found that on column methylation using m-tri ...uoromethylpheny trimethyl ammonium hydroxide, and diazomethane methylation gave the ... result, provided interfering trigylcerides and other neutral fats were removed from plasma samples by an initial hexane extraction. Haan et. al., (1979) have compared diazomethane methylation with methylation by methy iodide in dimethylformamide and obtained identical results.

D. Procedural Checks To Assure Reproducibility

The internal standard method appears the most accurate, except that it is crucial that the concentration of the added internal standard be accurately known. We have found that the standard acid can be quantitatively weighed and dissolved in methanol, stored at -40^0 in a reactivial with mininert value, and aliquots withdrawn to be added to samples on a daily basis or as necessary. The concentration of the standard is checked periodically by adding 10 µl to quantitative standards containing 16:0, 16:1, 18:1 and 18:2. Values differing by greater than 10% from those originally determined indicate changes in internal standard concentration or column efficiency.

We have also developed a method for monitoring day-to-day variations in extraction efficiency. This entails adding internal standard to freshly prepared aliquots of plasma, storing theset at -40^0, then extracting the standards daily or as required along with the samples of interest. A

drastic change in individual or total NEFA indicates column deterioration, microprocessor malfunction, or gross errors in extraction and derivatization. We find that plasma samples can be safely stored for at least a month and are quite useful as a procedural check.

Number of Gas Chromatographic Analysis of NEFA - December 1978 through July 1979

	Number of GC Analyses
Trauma patients (12/78 through 7/79) (total patients: 19)	167
Animal Studies	
Rats	377
Hamsters	71
Hyperalimentation Studies	98
Renal Disease Studies	14
Heparin Effects on NEFA and *in vitro* drug binding	125
NEFA levels in healthy volunteers of different ages, exercise and eating habits - required to develope normal standards.	222
Development of analytical procedure (standard mixtures, model plasma, methylation procedures, extraction procedures, solvent checks, etc.)	203
Miscellaneous (instrument calibration)	103
TOTAL	1407

II. Application to Fat Emboli

The method described is currently being used to determine individual and total NEFA levels in plasma from humans and experimental animals. To date we have found trends between NEFA levels and clinical symptoms in trauma patients. Correlation of the total free fatty acid level with the PO_2/FIO_2 ratio has been carried out in 19 trauma patients. Normal total free fatty acids in our laboratory are 110-120 mg%. Markedly elevated free fatty acid levels averaging 280 mg% are associated with PO_2/FIO_2 ratios averaging 1.9, indicating respiratory distress. With

a relatively normal total fatty acid level averaging 134, the PO_2/FIO_2 ratios are above 2.5. Statistical analysis of this is not possible with only 19 patients. Abnormally high levels of NEFA are observed within 24 hrs. post trauma and clinical recovery is associated with NEFA levels in a "normal" range that is significantly lower.

D. COLLABORATIVE STUDIES - DEPARTMENT OF PHARMACOLOGY

Following establishment of a reproducible qualitative fatty acid analysis, we have worked in collaboration with other departments interested in drug metabolism and their relationship to NEFA. Attached in the Appendix is a manuscript which has been accepted for publication in the British Journal of Pharmacology. This preliminary work will be valuable in future investigations in understanding albumin binding sites for free fatty acids and is an indication of the type and depth of collaboration possible in this institution.

I. Animal Studies

Results from the animal model studies of the effect of traumatic injury on drug metabolism are very encouraging. A considerable amount of time was expended in developing a suitable model for traumatic injury. Considerations concerning reproducibility have dictated that specific surgical procedures be utilized to institute traumatic injury. We have found abdominal aorta ligation to be a suitable primary model.

The following paragraphs are a summary of a paper in preparation which describes the reduction in both cytochrome P-450 and mixed function amino oxidase (MFAO) activities following traumatic injury. These two enzymes are the primary drug oxidizing enzymes in mammalian metabolism.

II. EFFECTS OF MODEL ISCHEMIC INJURY ON DRUG METABOLIZING ENZYME SYSTEMS

A. Introduction

The rational treatment of traumatic injury requires understanding of post traumatic drug metabolism. We have chosen to use an animal model system to determine if drug metabolizing enzymes are altered by traumatic injury. Reproducibility of injury is required for this study, thus a model was chosen which would lead to a consistent traumatic injury. For this investigation, we determined that arterial ligation fulfilled the above stated objectives.

Stallone et. al. have previously reported, using infrarenal aorta ligation to produce regional ischemia in dogs. However, their measurements were restricted to parameters involving pulmonary pathology. Sarfeh and associates studied hepatic dysfunction in rats following repeated deceleration injury in Noble-Collip Drums. They concluded that shock produced altered splanchic hemodynamics resulting in hepatic ischemia which led to dysfunction as measured by bile flow. Chung and Brown reported that hind limb ligation in the rat resulted in a 50% reduction of cytochrome P-450 in 1 hour.

We have found that in adult male rats, abdominal aorta ligation results in the rapid occurrance of significant decreases in cytochrome P-450 and mixed function amine oxidase. Recovery of enzymatic activity was found to occur in about 3-5 days post-trauma.

B. RESULTS

Cytochrome P-450. Cytochrome P-450 as measured by either content or activity was found to decrease significantly two hours post-trauma (model ischemic injury). Levels of cytochrome P-450 decrease to 68% of control level within 24-hours post-trauma and then returned to control levels within 80 hours post-trauma as shown in Figure 1.

Mixed Function Amine Oxidase. Mixed function amine oxidase levels as measured by enzyme activity was found to decrease in a fashion similar to that observed for cytochrome P-450, as illustrated in Figure 1. One significant observation from this study is that the rate of recovery from post-traumatic injury for mixed function amine oxidase is slower than that for cytochrome P-450.

C. DISCUSSION

The initial problem, which we have overcome, was the development of a satisfactory model for traumatic injury. Prior to instituting abdominal aorta ligation, femoral artery ligation and sciatic nerve resections were performed as model trauma. However, neither of these procedures were found to affect the hepatic enzymes cytochrome P-450 or mixed function amine oxidase. A shock model, consisting of removing 40-50% of the blood volume (via heart puncture) was also investigated and found to have no effect on either cytochrome P-450 or mixed function amine oxidase activities. However, abdominal aorta ligation was found to produce significant drops in both cytochrome P-450 and mixed function amine oxidase activities and content. In the older group of animals, abdominal aorta ligation only slightly altered the animal's ability to walk, and after a 24-hour recovery period, the animals appeared physically normal.

Both cytochrome P-450 and mixed function amine oxidase are important enzymes in drug oxidation. Cytochrome P-450 is known to oxidize a wide variety of drugs while mixed function amine oxidase has been reported to mainly oxidize drugs containing sulfur or basic amines. Thus, a significant decrease in the levels of these enzymes suggests the possibility of drug overdosing when dealing with drugs having a narrow therapeutic index. In addition, mixed function amine oxidase has also been implicated in protein synthesis. Many pathological events can be

hypothesized based upon trauma induced impairment of protein synthesis, however, these would be only conjecture at this time since we have no proof of impaired protein synthesis. We are presently investigating these possibilities.

Experiments have also been undertaken to measure the effect of trauma on glucuronly transferase. Preliminary results indicate that hepatic microsomal glucuronly transferase activity increases in response to ischemic injury.

Evaluation of animal serum NEFA levels following the ischemic injury correlate well with changes in cytochrome P-450 and MFAO activity. These increases in NEFA are similar to those found in multisystem injured humans both in magnitude and type. The preliminary data is encouraging as a basis to understand the pathophysiology of fatty acid toxicity. While no specific NEFA has been identified at the present as more toxic than others, with more qualitative analysis this is certainly possible to accomplish.

If these results can be extrapolated to humans, it implies that certain aspects of post traumatic drug therapy should be modified. Drugs which are inactivated by oxidative metabolism should be given in lower doses; drugs which are conjugated by glucuronly transferase and excreted should be given in higher doses; and drugs which are oxidatively metabolized to active forms which are in turn glucuronidated and excreted should be given in much higher doses.

III. EXPERIMENTAL CONSIDERATIONS

It has been necessary to surmount certain difficulties in obtaining statistically significant data for this project. Our experience with measuring cytochrome P-450 and MFAO in Sprague Dawley outbred rats has revealed that there is a certain variation in enzyme levels between

animals, and this variation is especially large for MFAO. This can be overcome by utilizing large numbers of animals in each experiment. Typically, we have had to use 16-40 animals for each data point to achieve a $p < 0.05$ (students t test). Although using larger number of animals has proved to be effective, it is expensive in both time and supplies (primarily animal costs).

An alternate approach is to utilize a population of animals which have more consistant enzyme levels. We have conducted one experiment using inbred (Fisher 344) rats and found that traumatic injury produced the same effects as with outbred (Sprague Dawley) rats but that the standard deviations for enzyme levels were somewhat better. We have also examined four strains of inbred mice and found that levels of cytochrome P-450 and MFAO are reasonably consistent between individuals of the same strain, but vary independently from strain to strain. This suggests that both enzymes are under genetic control and that the outbred rats probably have several alleles from the MFAO locus. In summary, inbred or F1 hybrid animals may prove to be a more cost effective model for our traumatic injury studies. However, the outbred animals with their large gene pool are considered by many to more closely mimic humans, and choices as to which animal is most appropriate for a given experiment must be made considering all these factors.

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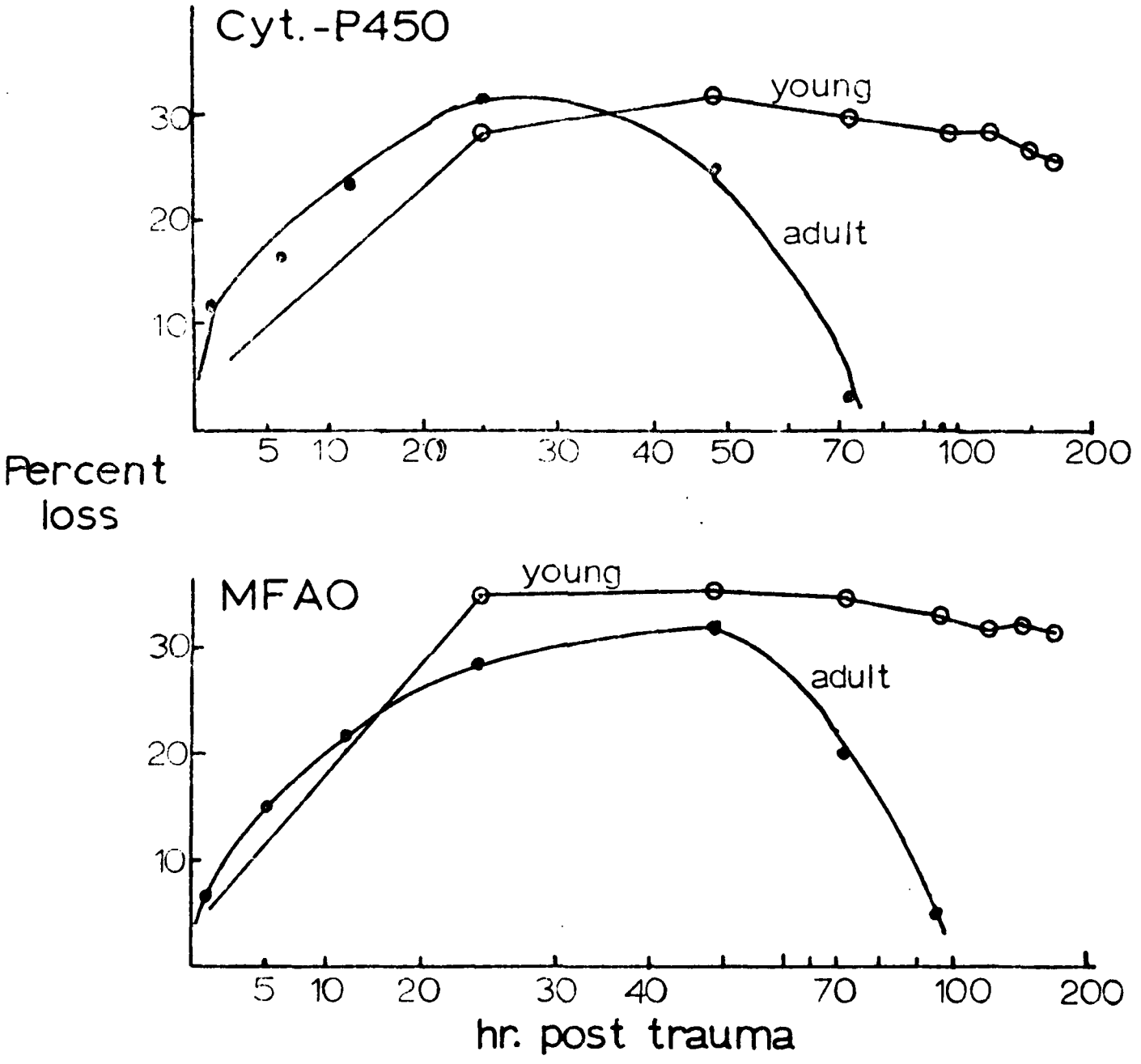

Figure 1

Effect of abdominal aorta ligation on cytochrome P-450 and mixed function amine oxidase (MFAO) activities in the rat liver. Measurements were made in vitro using microsomal preparations. Each point represents an average of approximately 18 animals.

Accepted by British Journal of Pharmacology

HEPARIN ADMINISTRATION INCREASES PLASMA WARFARIN BINDING IN MAN

There have been several recent reports that heparin administration can reduce the plasma binding of some drugs and hormones (Storstein and Janssen, 1976; Wood, Shand and Wood, 1979; Kessler, Leech and Spann, 1979; Desmond, Roberts, Wilkinson, Wood and Dunn, 1979; Thomson, Baird and Thomson, 1977). The effect of heparin administration on the plasma binding of warfarin is less clear. It was reported that *in vivo* elevation of non-esterified fatty acids (NEFA) by exercise or epinephrine administration markedly reduced the plasma binding of warfarin in rats (Gugler, Shoeman and Azarnoff, 1974). Since heparin also increases the plasma concentration of NEFA, it might be expected to produce similar changes in warfarin binding. In three human subjects studied by Nilsen, Storstein and Jacobsen (1977) however, intravenous administration of heparin (70 IE/kg) was associated with increased plasma binding of warfarin. The following study was performed to assess the effect of heparin on warfarin binding in a larger number of subjects and to examine the relationship between these changes and total and individual NEFA.

Heparin Sodium, 1000 USP units (Upjohn, Kalamazoo, Michigan) was administered to seven healthy young male volunteers 2-3 hours after a light breakfast. Blood samples were collected by direct venipuncture (immediately before and 15 minutes after the heparin injection) into glass tubes containing 10 USP units of heparin per ml of added blood. These were immediately centrifuged (at 4°C) and the plasma separated. Plasma warfarin binding was assessed after equilibrium dialysis of this fresh plasma against Sorenson's phosphate buffer (containing 0.5% w/v sodium chloride) adjusted to pH 7.4 in Teflon^{R} dialysis cells

at 37°C for 3 hours. ^{14}C warfarin (Amersham Corp., Arlington Heights, IL) was added to the buffer to achieve a concentration of 3 ng/ml and quench correction was performed using the external standards ratio method. The free fraction of warfarin in plasma was calculated as the ratio of absolute disintegration rates in buffer and plasma. Individual NEFA ($C_{16:0}C_{16:1}C_{18:0}C_{18:1}C_{18:2}$) were measured by a modification of the gas chromatographic method of Sampson and Hensley (1975) and "total" NEFA was calculated as the sum of these five individual NEFA.

The free fraction of warfarin in plasma fell by an average of 37% (range 28-45%) after heparin, that is from 0.0092 ± 0.0007 to 0.0058 ± 0.0008 (mean ± S.D.). The changes in inidividual subjects are illustrated in figure 1. These changes were accompanied by a rise in total NEFA from 83 ± 58 μMol.L^{-1} to 202 ± 96 μMol.L^{-1} (mean ± S.D. $p < 0.01$) in the seven subjects. No relationships were observed between the control individual or total NEFA concentrations and plasma free fractions of warfarin, however, or between the rise in total or individual NEFA and the fall in plasma warfarin free fractions.

Warfarin is almost exclusively bound to albumin in plasma and binds at two major sites. Enhancement of warfarin binding after heparin administration appears to be predominantly due to effects at the high affinity binding site and also occurs after *in vitro* addition of NEFA (Nilsen, Storstein and Jacobsen, 1977). The significance of this apparent lack of a relationship between changes in NEFA and in warfarin binding in our subjects and also in those of Nilsen, Storstein and Jacobsen (1977) is therefore unclear.

The potential clinical relevance of heparin's enhancement of warfarin binding in plasma is also unclear, although the two agents are frequently administered concomitantly during initiation of anticoagulant therapy. Since the pharmacological effect of warfarin is closely monitored and the dose adjusted accordingly during this period, a significant interaction due to enhanced binding is unlikely to be seen. Those undertaking pharmacokinetic studies which include the measurement of plasma binding of warfarin, however, should be aware of this phenomenon and collect blood samples accordingly.

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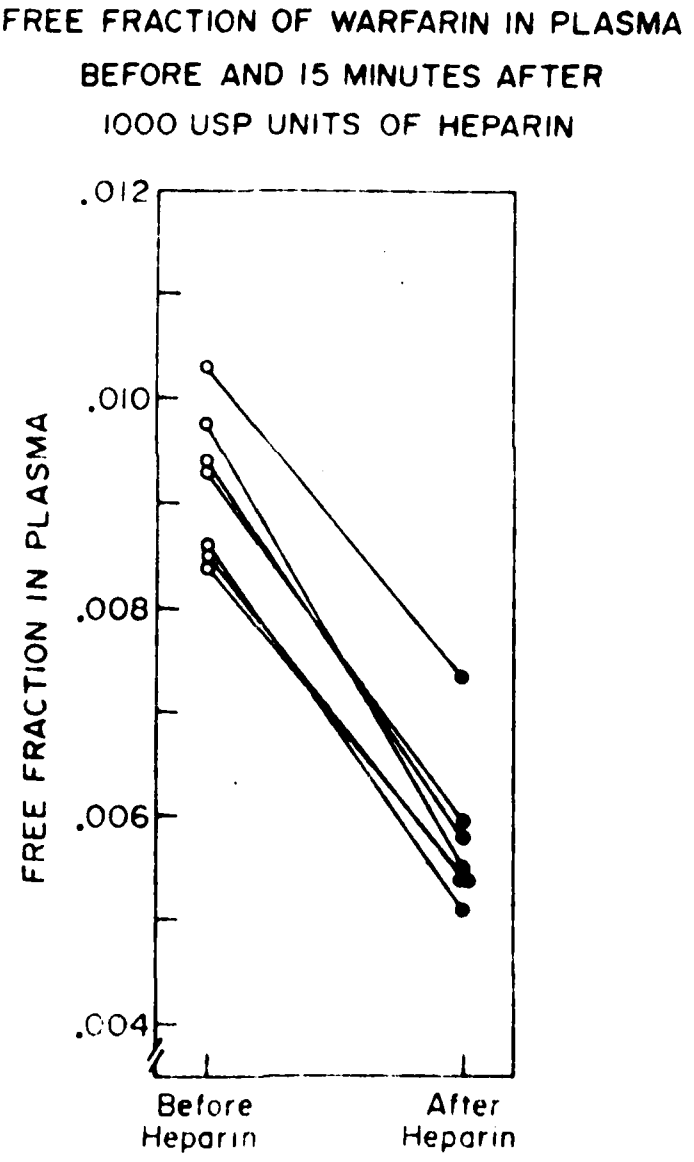
Figure 1. Free fraction of warfarin in plasma immediately before and 15 minutes after 1000 USP units of heparin intravenously.



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DRUG REDISTRIBUTION AFTER HEPARIN ADMINISTRATION: DIAZEPAM AND N-DESMETHYLDIAZEPAM

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Abstract

The effects of heparin 1000 U.S.P. units i.v. on the blood, plasma and free concentrations of diazepam and its metabolite, N-desmethyldiazepam have been investigated 3 hours after the oral administration of 10 mg diazepam to 5 normal volunteers. The % free diazepam and N-desmethyldiazepam increased at 15,30 and 60 minutes after heparin and the changes in free fraction correlated significantly with the change in non-esterified free fatty acids ($n = 0176$ $p < 0.002$ for both ligands). The free concentration of diazepam rose significantly from 3.6±1.04 to 6.9±1.33 ng/ml 15 minutes after heparin, while total blood concentration fell only from 144±54 to 130±57 ng/ml. Free concentrations of N-desmethyldiazepam rose from 0.62±0.17 to 1.01±0.34 and blood concentrations fell from 15±3.2 to 14±3.9 ng/ml.

A simple pharmacokinetic model suggests that tissue binding displacement may account for the greater than predicted rise in free diazepam concentration.

It is now recognized that the binding of diazepam is reduced in subjects given heparin[2]. The effect is an indirect one as it does not occur when heparin is added *in vitro*, and is generally assumed to involve the release of non-esterified fatty acids (NEFA). This suggestion is supported by the demonstration that NEFA can displace diazepam from albumin, its primary binding protein in plasma[1,2,15].

Although the effects of this interaction on the free fraction of a drug may be quite large, it is well recognized that the changes in free drug concentration *in vivo* may be much less dramatic[3,16]. This occurs because drug which is displaced from its plasma binding sites redistributes into tissues (including blood cells). This results in a fall in total plasma concentration which attenuates any rise in free drug concentration. We have therefore investigated the short-term effects of heparin administration on the circulating concentrations (total and free) of diazepam and its active metabolite, N-desmethyldiazepam.

Methods

Five normal male volunteers aged 23-40 received 10 mg of diazepam orally after a light breakfast. After 2¼ hours (i.e. during the post absorptive phase) a blood sample was obtained (time -15 min) and repeated 15 minutes later (0 time) after which 1000 U.S.P. units of heparin was administered and samples obtained at times +15, +30 and +60 min. All samples were obtained by separate venipuncture and transferred to all glass tubes containing 10 U.S.P. units heparin/ml of added blood. Heparin was shown not to influence diazepam or N-desmethyldiazepam binding *in vitro*.

Whole blood plasma concentrations of diazepam and N-desmethyldiazepam were measured by a GLC method[8] and plasma binding by equilibrium dialysis of duplicate 1 ml aliquots of plasma against Sorensen's buffer containing 0.5% sodium chloride (pH 7.4) in Teflon cells (Spectrum Medical Industries Inc., Los Angeles) for 3 hours at 37°C, at which time equilibration had been reached. In the case of diazepam, tritiated drug(4.4 ng, specific activity, 64.06 Ci/mole; purity > 98.5%) was added before dialysis. Following dialysis, two 300 µl aliquots of each plasma and buffer sample were added to 15 ml of Biofluor scintillation fluid (New England Nuclear, Boston) and the radioactivity determined using a liquid scintillation counter (Intertechnique SL 400). Quench correction was performed using the external standards ratio method and the percentage of free drug in plasma calculated as the ratio of absolute disintegration rates in buffer and plasma multiplied by 100.

Plasma binding of N-desmethyldiazepam was measured by addition of unlabelled drug to the buffer compartment in a concentration of 5 µg/ml. Drug concentrations in the plasma and buffer compartment were then measured at equilibrium by GLC[8]. Subsequent study using C^{14} N-desmethyldiazepam (specific activity 24.9 µ Ci/mg) confirmed that binding did not change with increasing drug concentration up to 5 µg/ml.

Plasma concentrations of non-esterified fatty acids were measured by a modification of the GLC method of Sampson and Hensley[12].

Students t-test for paired samples was used to assess the significance of changes in total and free drug concentrations, free drug fractions and NEFA between the T_0 and T_{15} minute values only. In all

cases, $p < 0.05$ was taken to be the minimal level of statistical significance.

Results

Effects on drug binding: The free fractions of both diazepam and N-desmethyldiazepam were increased 15 minutes after the heparin injection, and then began to fall (Table 1). NEFA also rose after heparin administration with a time course similar to that of the binding changes, such that there was a positive correlation between the percentage changes in NEFA and the percentage changes in free fraction of both ligands (Figs. 1 and 2).

Effects on circulating drug concentrations: It can be seen from Fig 3 that the concentrations of diazepam were falling prior to the heparin administration. After heparin, blood and plasma concentrations continued to decline apparently with the same half-life, and the blood/plasma concentration ratio remained at 0.63 - 0.64. It should be noted that at this time after diazepam administration, the early distribution phase is not yet complete[6] accounting for the apparent short half-life. In contrast, the free drug concentration rose at 15 minutes and then fell. This rise was statistically significant ($p < 0.01$; paired t-test), despite the fact that total drug concentrations tended to be falling somewhat. Of particular importance was the fact that there was no evident acceleration of the fall in total concentrations coincident with the obvious rise in free drug concentrations.

In contrast, the total concentrations of N-desmethyldiazepam remained relatively stable or even rose modestly prior to heparin (Fig. 4). After heparin, inspection of the data suggests a fall in total metabolite levels at 15 minutes, with a subsequent rise. Again, however, free N-desmethyldiazepam concentrations rose at 15 minutes and remained elevated thereafter.

Discussion

We have confirmed that heparin administration reduces the plasma binding of diazepam and have in addition shown that a similar, though somewhat less marked, change occurs in the plasma binding of N-desmethyldiazepam. The relationship between the changes in free drug fractions and those in NEFA are consistent with an indirect effect due to heparin-induced release of NEFA which displace these ligands from their binding sites (presumably albumin)[1,2,15].

Binding displacement interactions are clearly most complex and involve not only altered distribution, but also the potential for changes in elimination. In the present situation, there is the added problem that the changes in plasma binding are transient. For this reason we can only discuss the immediate effects of the interaction with any degree of confidence since any change in elimination (which will take a finite time to manifest, dependent on the elimination half-life) is likely to be minimal. In theory the immediate effect of drug displacement from plasma proteins should be a rapid redistribution

within the well-perfused central compartment, resulting in an obvious and abrupt fall in total plasma concentration,[3,16] an effect that has been shown for sulphonamides[10], tolbutamide[14] and bilirubin[11]. This fall in total concentration depends on the volume of distribution of the rapidly equilibrating tissues. It also attenuates any rise in free drug concentration which should therefore be of a lesser magnitude than the increase in free fraction. In the case of diazepam, however, the rise in free drug concentration (92%) was almost as great as the change in the % free fraction (140%). Furthermore, the total concentration of diazepam continued to fall along the same exponential slope without any obvious, sudden fall as has been noted for other compounds. The degree of discrepancy of the data from theory can be judged from the simple pharmacokinetic model of Gillette[3,17] (Fig. 5). The predicted data were derived by assuming that displaced drug would redistribute essentially instantaneously throughout the central compartment, which for diazepam has a volume of distribution (based on plasma concentrations) of about 20 l[6]. For our present purpose, however, we wish to consider extravascular redistribution so that the relevant volume should relate to whole blood. This modified volume of the central compartment can be calculated by dividing the above value for the central compartment by the blood/plasma concentration ratio of 0.64, giving a value of 31 l. According to Gillette, the volume of distribution of total drug in the body (Vd_B) can be visualized as:

$$Vd_B = V_B + \frac{V_T f_B}{f_T}$$

where V_B and V_T are the real volumes of blood and tissues and Vd_B

now represents the volume of distribution of the central compartment based on whole blood concentration. Using a value of 5 1 for V_B and calculating f_B at zero time we can solve for the term V_T/f_T, which is the volume of distribution of free drug in the rapidly equilibrating extravascular tissues and obtain a normal value of about 1000 1. If we assume that no tissue binding displacement occurs, then we can use the determined value of f_B 15 minutes after heparin to calculate Vd_B at that time. This was found to be 67 1. Recognizing that drug concentration is the inverse of volume, then the total drug concentration should have fallen by 31/67, i.e. 45% of the value that it would have been without heparin (given by extrapolated line in Fig. 5). The theoretical effects on free drug concentration can be calculated on the basis of the volume distribution of free drug (Vd_F) as

$$Vd_F = \frac{V_B}{f_B} + \frac{V_T}{f_T} = \frac{Vd_B}{f_B}$$

In this case, free drug concentrations should barely have risen at all (Fig. 5), and certainly would not have resulted in such an obvious rise as was the case. On this basis, we believe the data suggest that heparin administration displaces diazepam from its extravascular tissue binding sites as well as from plasma binding sites. Furthermore, it appears that tissue displacement is far more important in producing the rise in free drug concentration than plasma displacement. It should be stressed that all these calculations are approximate. However, in their use we have attempted to maximize the theoretical

increase in free drug concentration by (1) choosing the volume of distribution of the central compartment rather than Vd_{β} or Vd_{ss}, and (2) ignoring the effects on elimination which would tend to lower free drug concentration by enhancing elimination, and (3) assuming that redistribution takes place rapidly. All these three considerations appear pharmacokinetically reasonable in the present context.

The data for N-desmethyldiazepam are somewhat different in that concentrations were steady or even rising during the control period. This is consistent with the long time needed for this metabolite to reach its peak concentration (at about 24 hours)[6]. Following heparin, free drug concentrations rose 63% in association with a 100% increase in free fraction. There was also a suggestion of a small, transient fall in whole blood and plasma concentrations which would be theoretically consistent. Unfortunately, there are no data available concerning the volume of distribution of the central compartment of N-desmethyldiazepam since pharmacokinetic studies have only examined the oral route[7]. Thus we cannot use the above model to test whether these changes are consistent with plasma binding changes alone, as was done for diazepam. All that one can say is that the direction of the changes is appropriate, although the magnitude of the rise in free drug again raises the issue of tissue displacement.

The pharmacokinetic events occuring after immediate displacement are likely to be complex and potentially involve slower redistribution with deep, peripheral pools of drug, as well as altered elimination, which in turn depends on the drug in question. Because of this and because the observed effects of heparin are transient, we should be

cautious about over-interpretation of the present data after 15 minutes. We might predict, however, that with both these compounds whose hepatic elimination is relatively inefficient and probably restricted by drug binding in blood, total clearance would increase with reduced binding. This was not observed with diazepam over the short period of observation in this study, probably because of the transient nature of the displacement and because it takes several half-lives for changes in total clearance to reach a new pseudoequilibrium. The final outcome of continued heparin administration remains to be elucidated, but any changes in free drug concentration would likely be transient due to the compensatory fall in total drug concentration, as the new steady-state is achieved[17].

Clinically, several opportunities exist for this interaction to occur because heparin is often given during procedures, such as cardiac catherization and open heart surgery, to patients under diazepam sedation. However, the outcome will depend on whether free diazepam (and N-desmethyldiazepam) concentrations are related to the drug's effects. While the increased incidence of adverse reactions to diazepam in hypoalbuminemic patients[4] is consistent with increased free drug concentration, the relationship between diazepam's effects and its circulating concentrations is unclear[8,9,12] and confounded by the development of tolerance[5]. Furthermore, the present suggestion that tissue binding displacement may occur with heparin administration raises the real possibility of displacement from the drug's sites of action. Clearly, the pharmacodynamic aspects of this interaction require investigation before we can fully assess its clinical relevance.

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Figures

Fig. 1: The relationship between the changes in free diazepam fraction and non-esterified free fatty acids (NEFA).

Fig. 2: The relationship between the changes in free N-desmethyldiazepam fraction and NEFA.

Fig. 3: The effect of heparin (H) on plasma, blood and free concentrations of diazepam.

Fig. 4: The effect of heparin (H) on plasma, blood and free concentrations of N-desmethyldiazepam.

Fig. 5: Observed (solid symbols) and predicted (open symbols) effect of heparin on diazepam kinetics.

Table 1: Effect of heparin on the percentage free diazepam (D) and N-desmethyldiazepam (DMD). Values are mean ± standard deviation.

Time, min.	-15	0	+15	+30	+60
% free D	1.59±0.28	1.66±0.35	3.99±1.88*	3.39±1.87	2.94±1.57
% free DMD	2.30±0.61	2.50±0.65	5.00±1.96*	4.20±1.84	3.60±1.68

Statistical significance between values obtained at times 0 and +15 min.
*$p < .05$

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Figure 1

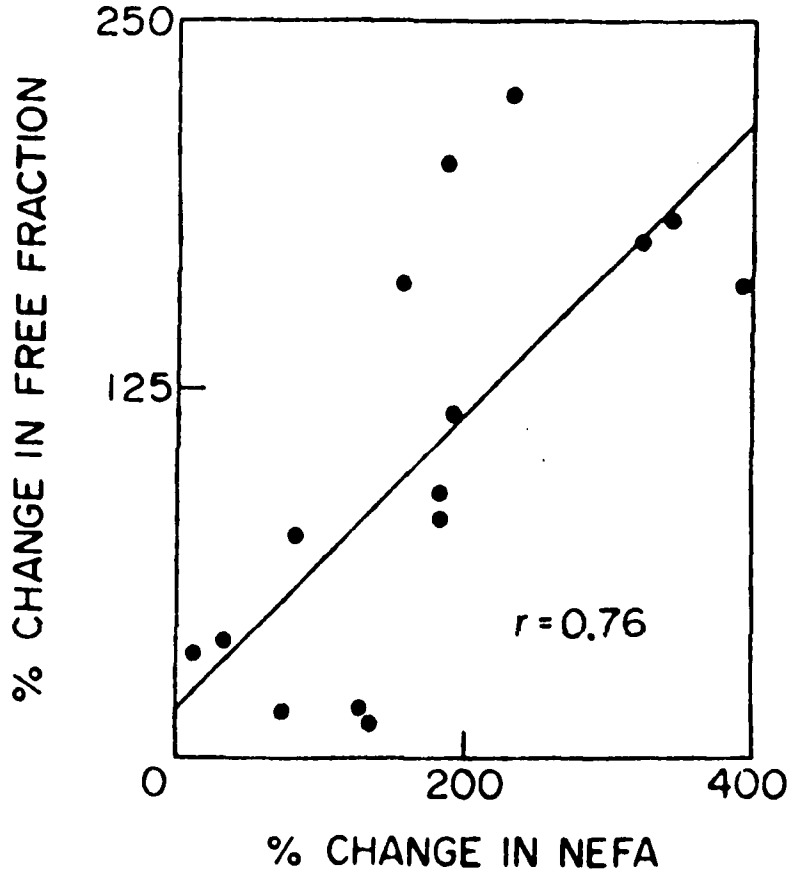


Figure 2

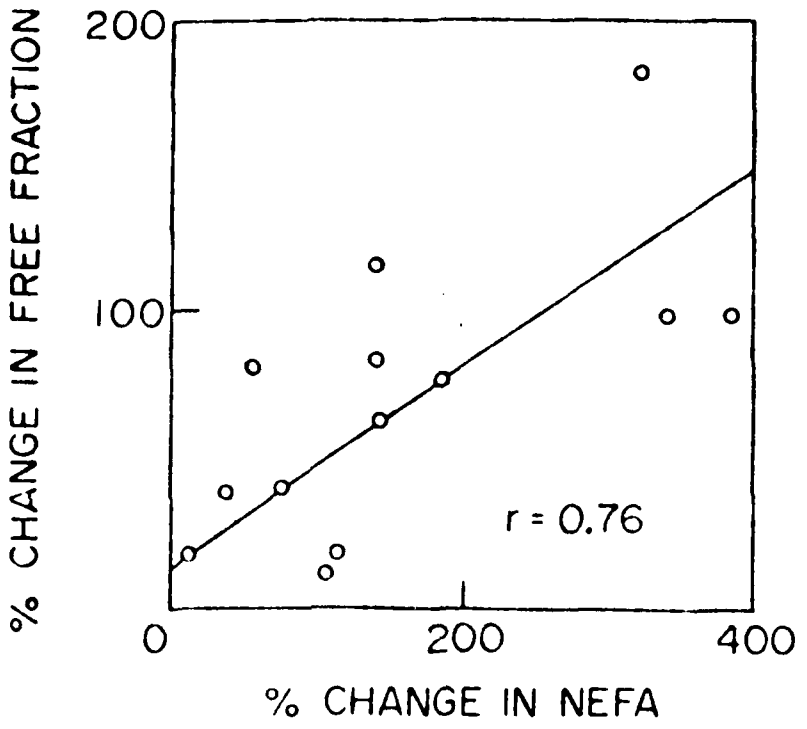
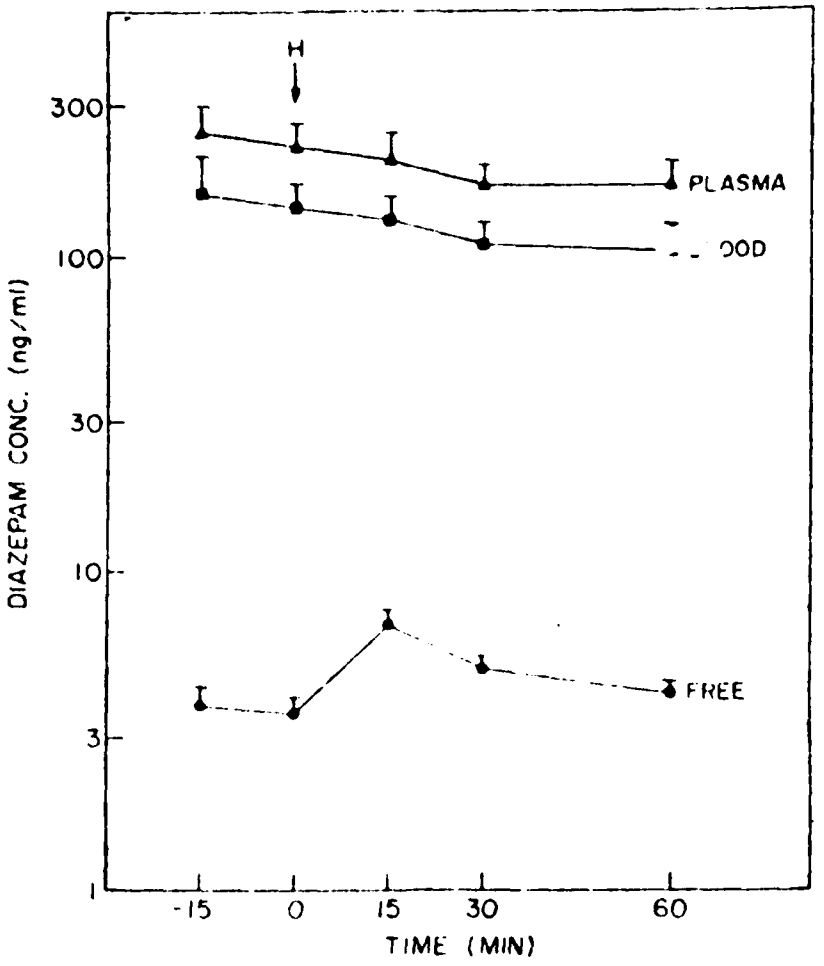


Figure 3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TIME (MIN)

Figure 4

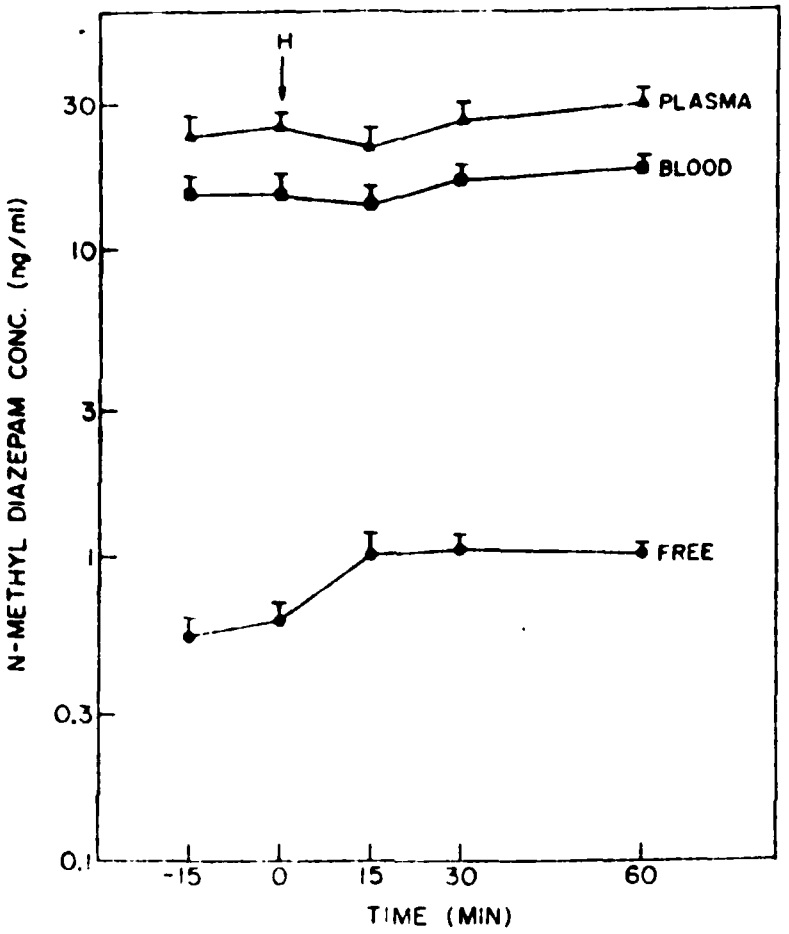
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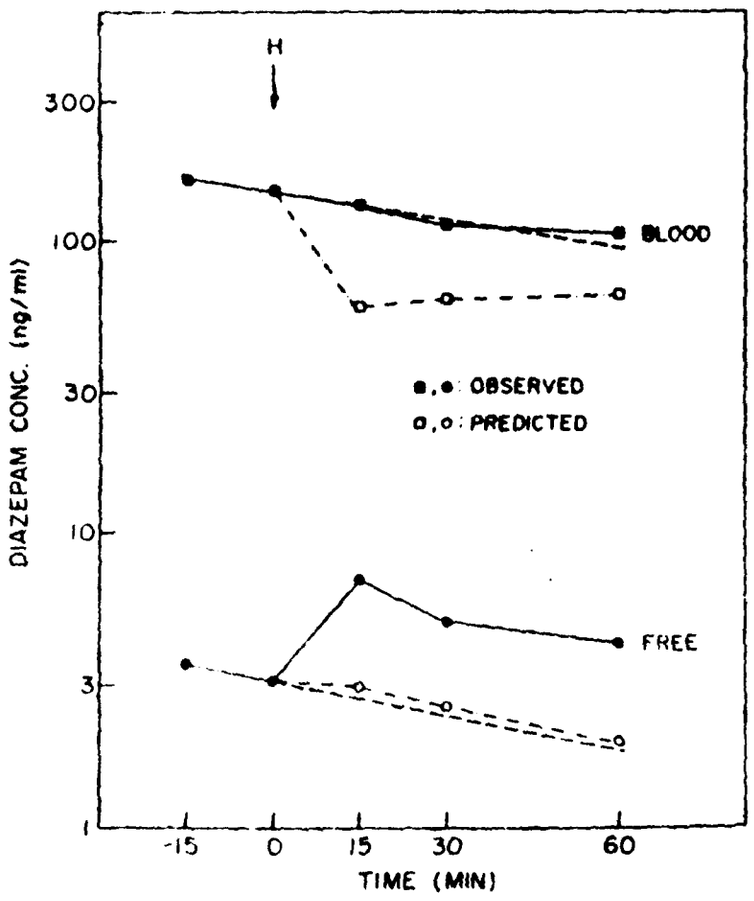
Figure 5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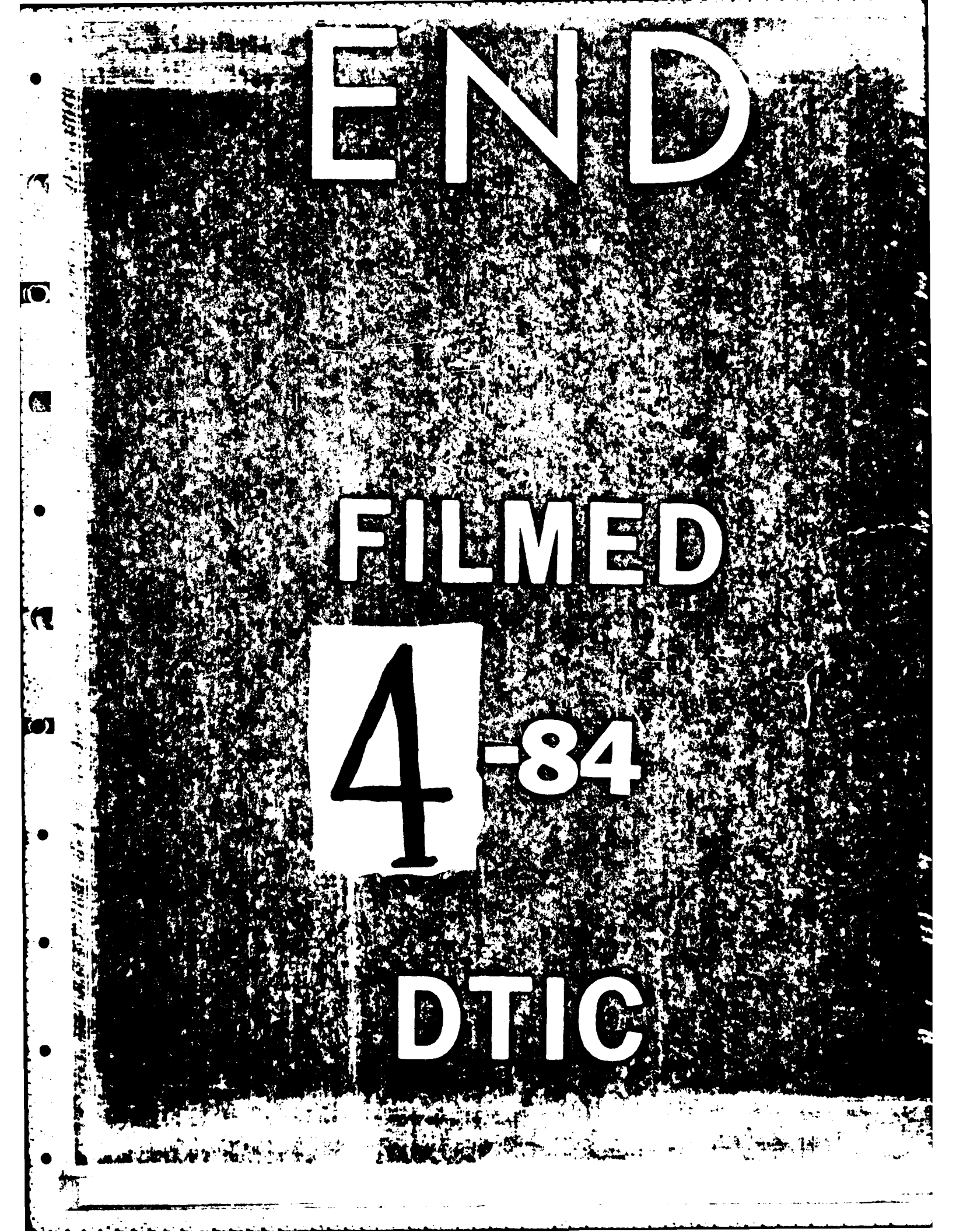
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